Research Progress Report

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Freeze-Dried Human Red Blood Cells
Contract No. N00014-90-C-0053

October 15, 1992

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RESEARCH PROGRESS REPORT
SUBMITTED TO THE US NAVAL MEDICAL RESEARCH AND DEVELOPMENT COMMAND
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SUMMARY

This research progress report summarizes our progress in basic red blood cell research since our last progress report submitted to Naval Medical Research and Development Command on July15, 1992.

As outlined in the summary report from July 15, 1992, a preliminary clinical evaluation of in vivo circulation of autologous, lyophilized and reconstituted human red blood cells was undertaken to establish a baseline level of in vivo performance of lyophilized red blood cells from both human volunteers. The cell survival results demonstrated that reconstituted, lyophilized red blood cells remained in circulation in vivo for the same period as normal non-lyophilized red blood cells.

Since our last Progress Report of July 15, 1992, our basic research activities have focused on the following : (1) determination of the relationship between residual moisture and properties of lyophilized red blood cells; (2) Evaluation of the stability of lyophilized red blood cells at +4°C and -20°C; (3) Supplementing our present buffer formulations with additives that may prevent degradation of lyophilized red blood cells during extended storage at +4°C; (4) effects of lyophilization on surface antigens of human red blood cells; Effects of sample configuration on stability of lyophilized red blood cells; (6) Reconstitution of lyophilized red blood cells with polymer free washing solutions.
Some of the above projects are still ongoing , however, preliminary results show that:

- Lyophilized human red blood cell scan be stored at +4°C for at least 49 days without any major alteration in both the biochemical and biophysical properties of the cells.
- Using Arrhenius kinetics we are able to predict the storage shelf life of lyophilized red blood cells at +4°C to be at least 6 months.
- Lyophilized red blood cells can be reconstituted with polymer free reconstitution salt solutions.
- Lyophilization buffers containing boosting components such as phosphate, inosine, glucose, pyruvate and adenine (PIGPA) appeared to improve the overall recovery of lyophilized red blood cells after extended storage period at +4°C.
- Red blood cells surface antigens are not altered by our lyophilization process.

We have also been evaluating different rehydration procedures that will eliminate the need for washing the lyophilized red blood cells upon rehydration. In our last Progress report of July 15, 1992, we indicated that our lyophilized red blood cells can be rehydrated with a "Two-Step" procedure involving an initial rehydration with a diluent followed by centrifugation to remove supernatants. This washing procedure so far has remained the most effective in maintaining cell qualities that are comparable to that of fresh non-lyophilized red blood cells. Experiments are still ongoing in this area to develop a buffer formulation that will allow a "No Wash" procedure.

UPDATE OF PROJECT STATUS RELATIVE TO 1989 MILESTONES

Cryopharm submitted its original research proposal on lyophilized human red blood cells in September, 1989. In that proposal we included a chart of research milestone and a copy of that chart is included in this report. In our Progress Report of November 1991, January 1992 and April 1992, we outlined all our progress to date with respect to the proposed projects and we showed that most of the proposed studies have been completed at the specified projected dates.

Our final 1989 milestone proposed the filing of an IND for Phase I clinical trials at the end of Year 3 (May 1993). We believe that our project remains on target. We have already successfully conducted low dose autologous human red blood cells in vivo survival studies with four healthy volunteers in collaboration with the Department of Biomedical Research at Tufts University School of Medicine. These studies have been conducted with the approval of the appropriate Institutional Review Board. The design and dosage of these studies parallels what would be used in any Phase I clinical trial. We have demonstrated the safety of the lyophilized product in four healthy volunteers as reported in the present Progress Report. With our present successful clinical trial, we anticipate filing for IND at the projected date.

In June 1992, CryoPharm held a preliminary IND meeting with the Food and Drug Administration (FDA) to identify the appropriate regulatory pathway for its blood cryopreservative additive solutions which can be used for both lyophilization and freezing of human red blood cells. CryoPharm received a direct validation of its regulatory strategy to conduct limited clinical trials designed to show adequate in vivo cell survival of preserved red blood cells. FDA concurred with the Company's position that the cyoprotectant components are substantially safe, and that the efficacy of the preserved cells could be sufficiently demonstrated by a limited survival test program under an IND approval. FDA also indicated that the cryopreservative solutions could be regulated as either a device or a drug, which would enable the company to pursue interim contract GMP manufacturing, pending approval of its own facility (this option for OEM production would not have been available with a biologic product). Based on FDA's input, the Company plans to file for an IND for its red cell solution during 1993.

RESEARCH PROGRESS REPORT

Background

In the July 15, 1992, Progress Report we reported that Cryopharm's basic red cell research had developed an improved lyophilization buffer formulation that allowed us to successfully lyophilize human red blood cells. The reconstituted cells exhibited properties that are similar to that of normal fresh non-lyophilized red blood cells . In addition, autologous chromium labeled reconstituted lyophilized red blood cells were infused into 4 healthy volunteers during Cryopharm's third clinical study in April-June 1992. Detailed report from this in vivo survival study was reported in our July 15 Progress Report .

2. Basic Red Cell Research

In the Future Plans section of our July 15, 1992 Progress Report we proposed to carry out preliminary storage stability studies to determine the shelf life of lyophilized human red blood cells at storage temperature of +4°Celsius and higher. The stability of lyophilized preparations during prolonged storage can be predicted quantitatively from results of short term degradation studies at elevated temperatures. This procedure is based on the observation that some measurable quality of the cells such as deformability, filterability and overall cell recovery will decline linearly with time at the selected temperature. The rate of degradation of the lyophilized product can be determined from the Arrhenius relationship for thermal degradation. The theory and the associated equations for calculating the shelf life of lyophilized preparation have been presented in our April 15, 1992, Progress Report. Our research activity in this area was directed initially at understanding the relationship between residual moisture contents of lyophilized red blood cells and cell quality. By using this approach we wanted to determine the maximum level of dryness the red cell s can sustain without any major alteration in cell properties. In the second phase of our storage stability data, the residual moisture was fixed at the maximum tolerable level for lyophilized human red blood cells. By fixing the residual moisture content of our lyophilized red cell we can now monitor the stability of the cells at storage temperature of +4°Celsius over an extended period.

Relationship Between Residual Moisture and Weight Loss

Red blood cells were mixed with standard lyophilization buffer (Buffer #1, was used for the in vivo survival study) at a blood to buffer ratio of 1:3. The red cell :buffer mixture was lyophilized at -25°C to different residual moisture contents. Residual moisture contents were measured using previously described method(Karl Fischer, reference #1). In Figure 1, we show the relationship between weight loss and residual moisture content. The results show that there is a linear relationship between residual moisture and the apparent weight loss. For subsequent studies the weight losses of

lyophilized samples were measured while the residual moisture can be derived from the weight loss based on the linear relationship between the two parameters.

Relationship Between Weight Loss and Quality of Reconstituted, Lyophilized Red Cells

Red blood cells were mixed with standard lyophilization buffer (Buffer #1) at a ratio of 1:3 and then lyophilized according to standard procedures. Lyophilized red blood cells with different weight losses were rehydrated with CryoPharm Reconstitution buffer (Reconstitution Buffer #1) and then washed in isotonic dextrose saline. Red cell deformability profiles were measured with the ektacytometer (reference 2) and filterability by gravity driven-filtration device (reference 3). In Figure 2, we show that the maximum level of weight loss the red cell preparation can sustain without any major alteration in cell properties is 35%, using the present standard lyophilization buffer formulation.

Using Arrhenius Kinetics to Determine the shelf life of lyophilized Red Blood Cells

Red blood cells were mixed with standard lyophilization buffer (Buffer #1) as previously described and then lyophilized at -25°C such that the final weight loss is equal to or less than 35%. The lyophilized samples were placed inside a blood bank refrigerator at +4C for 59 days. During this storage period all the normal cell indices were measured. Rheological parameters as well as the overall recovery were used as cell qualities that are most affected by extended storage. The rheological parameters are the most important properties of the red cells. Normal rheological properties are required if the lyophilized red blood cells are to carry out their normal physiologic function of oxygen delivery. The overall recovery gives an indication of the stability of the red cell membrane when suspended in isotonic solution. Using the Arrhenius equation in combination with the above cell properties, the shelf life of the lyophilized red blood cells can be predicted with a high degree of confidence. The above lyophilization procedure was repeated with a modified lyophilization buffer (Buffer #2). Buffer #2 contained chemicals that have been shown to boost the levels of essential glycolytic intermediates during storage at +4°Celsius.

Figures 3 and 4 show the relationship between cell properties and storage duration. The maximum shelf life at which the lyophilized samples maintained 70% of normal cell properties is 75 days for RBC in Buffer #1 and 300 days in Buffer #2. In using Arrhenius kinetics to predict the above shelf lives, the storage data generated at +4°C were fitted to a temperature dependent model generated using three different storage temperatures (-25°C, -10°C and +4°C). These preliminary storage stability data suggest that lyophilized samples can be kept at +4°Celsius for about 6 to 12 months at +4C. Note that this storage duration at +4°C is considerably higher than that for liquid stored red blood cells. Figures 5 and 6 show some preliminary results with samples that were stored at temperatures lower than +4°C. These results suggest that lyophilized red

blood cells can be stored at -20°C for at least 2 years without any major alterations in cell properties, Figure 5.

In addition to the above storage stability studies several experiments have been carried out to : (1) determine the preservation of surface antigens during lyophilization; and (2) simplify the formulation of our present reconstitution buffer.

Preservation of Selected Antigens on the Surface of Lyophilized Red Blood Cells

The main objective of this study was to determine the preservation of red cell surface antigens following lyophilization. Four fairly weak red cell antigens (P, N, Le^a and Fy^a) were selected for this study. These antigens are very labile, therefore, we assumed that their preservation would be good indication of overall antigen preservation during freeze-drying. Packed red blood cells were washed in isotonic dextrose saline according to standard centrifugation procedure. Washed red blood cells were mixed with standard lyophilization buffer (Buffer #1) at a blood to buffer ratio of 1:3. Packed RBC concentrates were prepared and lyophilized at -25°Celsius until the final weight loss was about 35%. Testing of surface antigens were carried out using standard test tube agglutination assay (reference 4). Results in Tables 1A-1E show that the above red cell antigens are preserved on the surface of the red blood cells following lyophilization and storage at +4°C for 4 weeks.

Rehydration Procedures

The present reconstitution buffer has been modified so that lyophilized red blood cells could be easily rehydrated with buffers that are less expensive and easier to prepare than the current formulations. The present reconstitution protocol still involves two washing steps. Research activities are still ongoing to develop either a "One Wash" or "No Wash" procedures.

Container Design:

CryoPharm has developed a successful working sterile plastic container for lyophilization. The working prototype has been tested in all our clinical studies. In each of the clinical study, we cultured samples of the rehydrated autologous red blood cells in media specified for sterility testing in Title 21 of the Code of Federal Regulations (CFR) and according to the procedures of the American Association of Blood Banks (AABB). All tests showed no detectable bacterial growth after seven days. CryoPharm is in the process of evaluating different modifications of the above bag designs with the goal of improving lyophilization efficiency. Some of the materials we are currently evaluating include the use of Teflon fabrics (which are expanded polytetrafluoroethylenes --- PTFEs). Preliminary results from experiments using PTFEs based bag designs are

comparable to that obtained with our standard plastic lyophilization bag (overall cell recovery, hematological cell indices and biophysical properties). PTFEs bags are however, less bulky and much easier to use than the plastic bags. Research in this area is still ongoing .

FUTURE PLANS

Results from the in vivo survival study (Progress Report July 15, 1992) are very encouraging in that it demonstrates for the first time that small doses of reconstituted, lyophilized red blood can be infused into healthy autologous donors with maintenance of normal physiologic functions . However, future studies will need to address the following issues:

1. Upgrade of Lyophilization Facilities to Conform to GMP Standards

CryoPharm proposes to upgrade our existing lyophilization facility to conform to GMP standards as required by the FDA for product licensing. Currently CryoPharm operates six research grade shelf lyophilizers, four of which are solely dedicated to research and are used for cycle development and evaluation of lyophilization parameters. The remaining two lyophilizers are placed in a Class 100 processing facility for small scale production of lyophilized red blood cells for in vivo survival studies under GLP conditions. All the lyophilizers in the "Clean Room" are not steam sterilizable and are only capable of research scale production of lyophilized products. These units cannot be upgraded to meet GMP specifications. Currently, our Class 100 clean room facility incorporates (a) a buffer preparation area, (b) a blood processing area, and (c) a lyophilizer area. In order to conform to GMP standards, CryoPharm proposes to upgrade the existing facility such that preparation of lyophilization and reconstitution buffers and processing of blood are done at separate locations to minimize the risk of cross contamination. CryoPharm proposes to carry out the following modifications to the existing facilities:

1. Buffer Preparation Area: All lyophilization buffers are presently sterilized by an aseptic filtration method. In order to assure sterility of our lyophilization buffers, CryoPharm expects to install a sterilizer for large scale terminal sterilization of lyophilization and reconstitution buffers. CryoPharm has already started to source the appropriate sterilizer along with required accessories (steam generators, equipment for production of water for injection quality) and will cover the cost of upgrading the current buffer preparation facility from private funds. This area will comprise a second GMP plant. The existing facility, once upgraded to GMP specifications, will be solely dedicated to blood lyophilization. CryoPharm expects to initiate extensive studies to:

(a) Develop the appropriate sterilization cycle for terminal sterilization of all processing solutions. Appropriate tests will be designed to determine the viral kill for the final

sterilization cycle such that it meets the minimum standard requirement of 8 log reduction in viral titre as requested by the FDA.

(b) Determine whether there is any adverse interaction between the chemicals in our lyophilization buffers and PVC buffer containers. Studies will be designed to detect release of any toxic materials or leachables. CryoPharm proposes to conduct storage stability studies on the lyophilization buffers at different storage temperatures (+4° Celsius, +37°Celsius) for a period of six months. The storage stability studies will define the storage conditions for our lyophilization buffers.

2. Pilot GMP Lyophilizer: CryoPharm proposes to install a steam sterilizable GMP grade lyophilizer capable of large scale production of lyophilized human red blood cells. This GMP lyophilizer will draw upon a clean steam source being installed as part of CryoPharm's. Once the lyophilizer is installed, we will conduct detailed evaluations of lyophilizer parameters (pressure, shelf temperature, sample temperature and shelf ramping rates) to scale-up the lyophilization cycle already developed for successful lyophilization of human red blood cells in our research. The resulting dried cells will be evaluated for cell quality using the following in vitro parameters: cell recovery; mean cell volume-MCV, mean cell hemoglobin-MCH, mean cell hemoglobin concentration-MCHC; hemoglobin species, glycolytic intermediates-ATP, 2,3-DPG, Lactates, oxygen carrying property, and cell morphology). These studies will begin once the clean room facilities have been developed to GMP standards.

3. Clean Room: The existing 600 square foot Class 100 clean room will be expanded to occupy, 1200 square feet of space with adequate humidity and temperature control systems to meet GMP standards. A steam sterilizable, GMP grade pilot lyophilizer will be installed as part of this facility. Since the existing clean room has no steam capability, sources of cooling water, drainage and steam venting must be installed as part of the facility upgrade. In addition, the existing HVAC system must be upgraded to deal with the increased heat load and to maintain proper room temperature and humidity levels.

Once the above modifications have been completed, CryoPharm proposes to conduct GMP trial runs to achieve all specifications for reconstituted lyophilized red blood cells. These trial runs are designed to test and document the effectiveness of red blood cells collected and lyophilized using CryoPharm's lyophilization procedures. These studies will also demonstrate whether lyophilized storage of red blood cells using CryoPharm's technology gives, a post-reconstitution product comparable to red blood cells stored in other available systems. Standard operating procedures (SOPs) will be finalized for all stages of the lyophilization process.

4. Cycle development and Container design: Currently, the present lyophilization procedures can accommodate a clinical size sample of about 160g of blood buffer mixture. Modifications to the container as well as to the lyophilization cycles are required in order to successfully lyophilize full units of blood. A proprietary container is

presently being evaluated with the hope that this will allow a more efficient lyophilization of human red blood cells.

5. Storage Stability Studies: We have started various experiments some of which are still ongoing to determine the shelf life of our lyophilized red blood cells. Preliminary results are very encouraging but also suggests some changes in membrane properties after extended storage at +4°C. The main goal of our research activities in this area is to determine the nature of the biochemical and biophysical mechanisms that are responsible for cell damage during storage at +4°C. Preliminary results with lyophilization Buffer #2 showed that certain additives can increase shelf life of lyophilized RBC. Understanding the mechanisms by which Buffer #2 protects RBC from storage induced cell damage will be the key to our developing processing conditions that will extend the current shelf life.

6. Determination of the relationship between storage stability, residual moisture and in vivo survival. Results presented in this progress report suggest that there is an optimum level of dryness that the red blood cells can sustain, above which cell damage occurs. Part of our research efforts will be directed at designing experiments that will allow detailed evaluation of the effects of residual moisture on in vivo survival and oxygen carrying capacity of red blood cells. In vivo survival of red blood cells dried to different levels of residual moisture will be assessed using animal models.

7. Simplification of the washing procedures. The current rehydration protocol involves two washing steps with dextrose saline. The ultimate goal of our research in this area is to eliminate the need for washing. Experiments will be designed to determine the in vivo survival characteristics of lyophilized red blood cells that have been reconstituted with different wash protocols using animal models.

Figure 1: Relationship Between Residual Moisture & Weight Loss

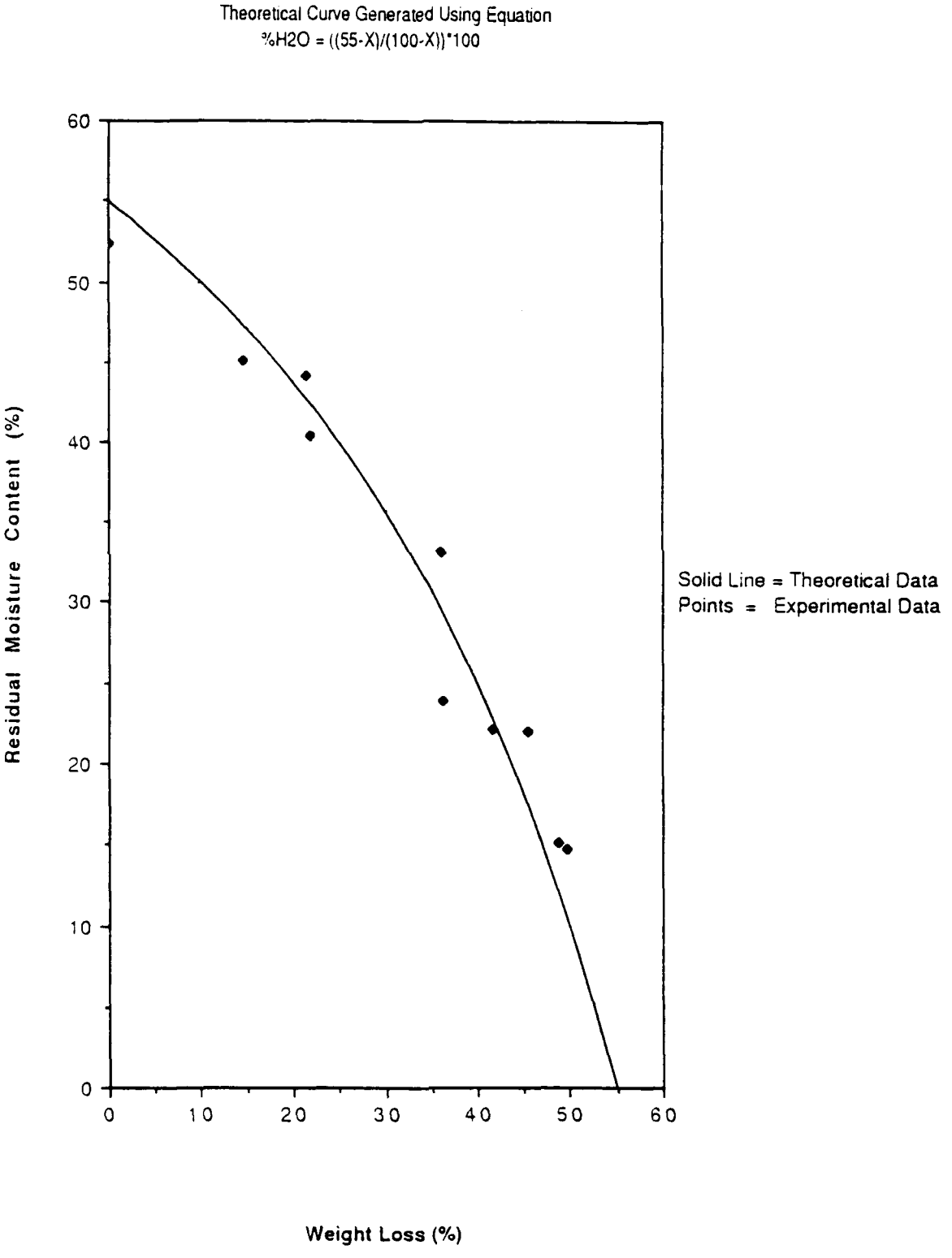

Figure 2: Relationship Between Weight Loss And Cell Quality

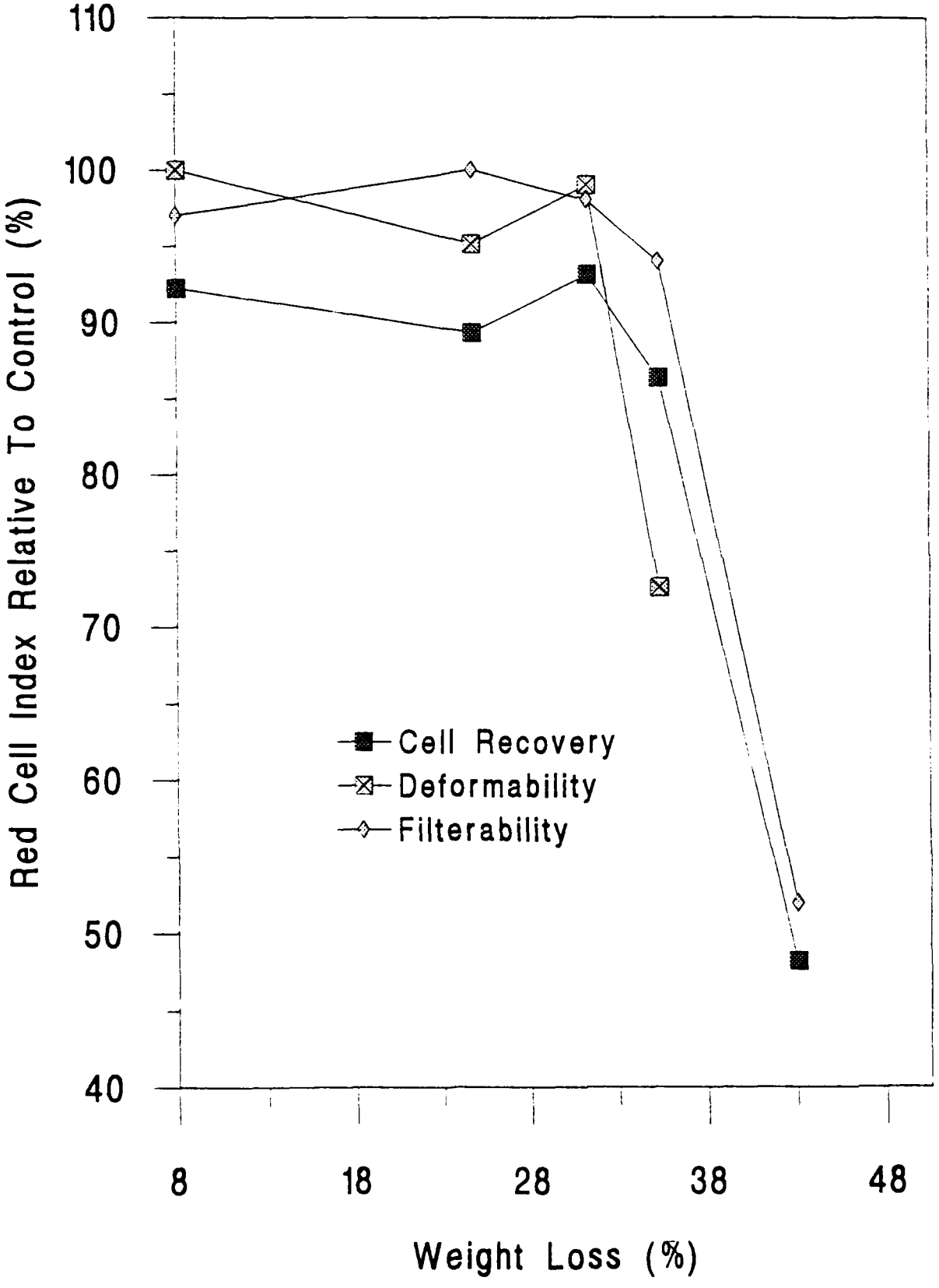

FIGURE 3

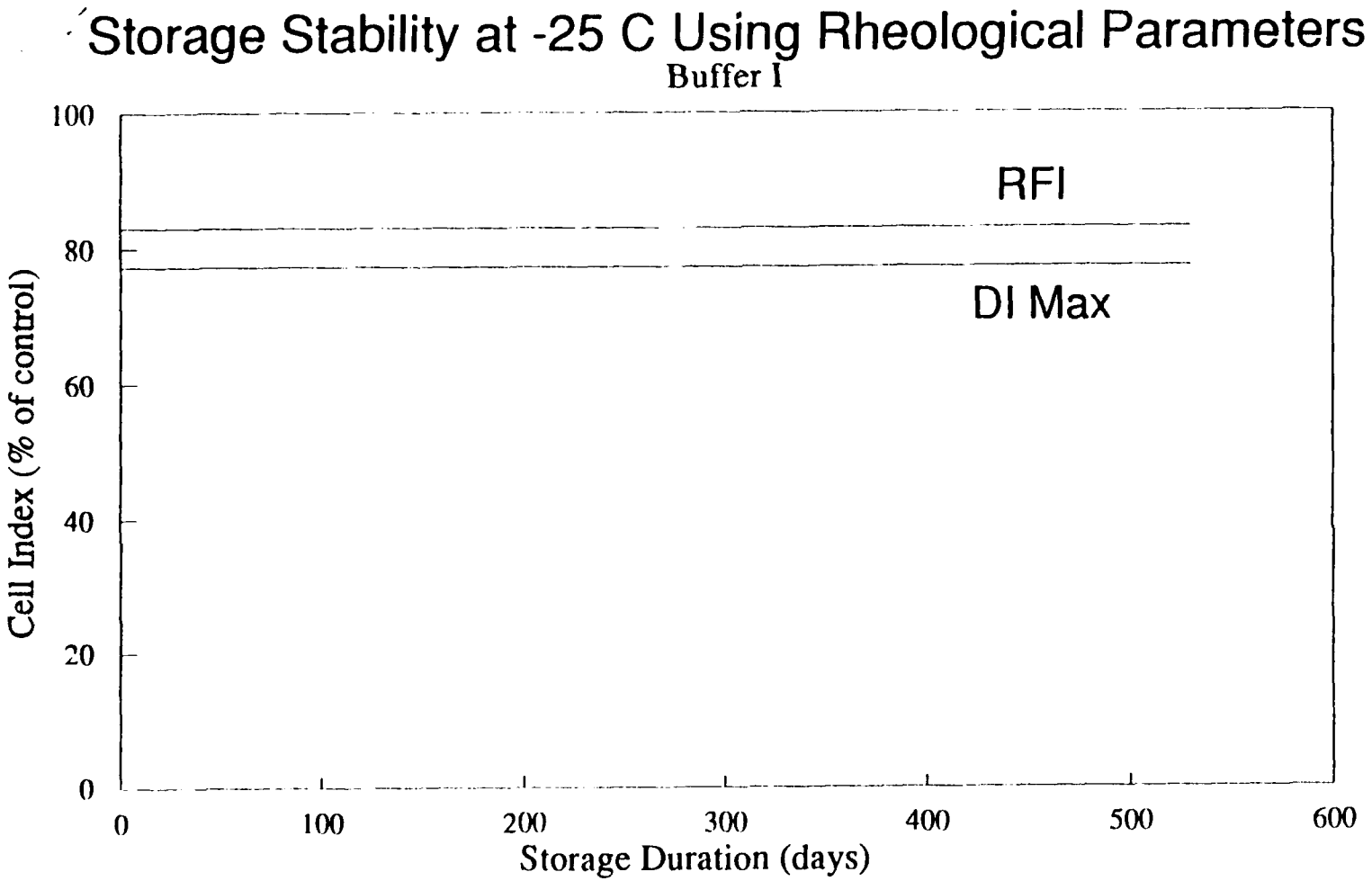
Storage Stability at -25 C Using Rheological Parameters
Buffer I
RFI
DI Max
Cell Index (% of control)
100
80
60
40
20
0
0
100
200
300
400
500
600
Storage Duration (days)

FIGURE 4

Storage Stability at +4 Celsius Using Both Rheological Parameters and Overall Cell Recovery

Buffer II

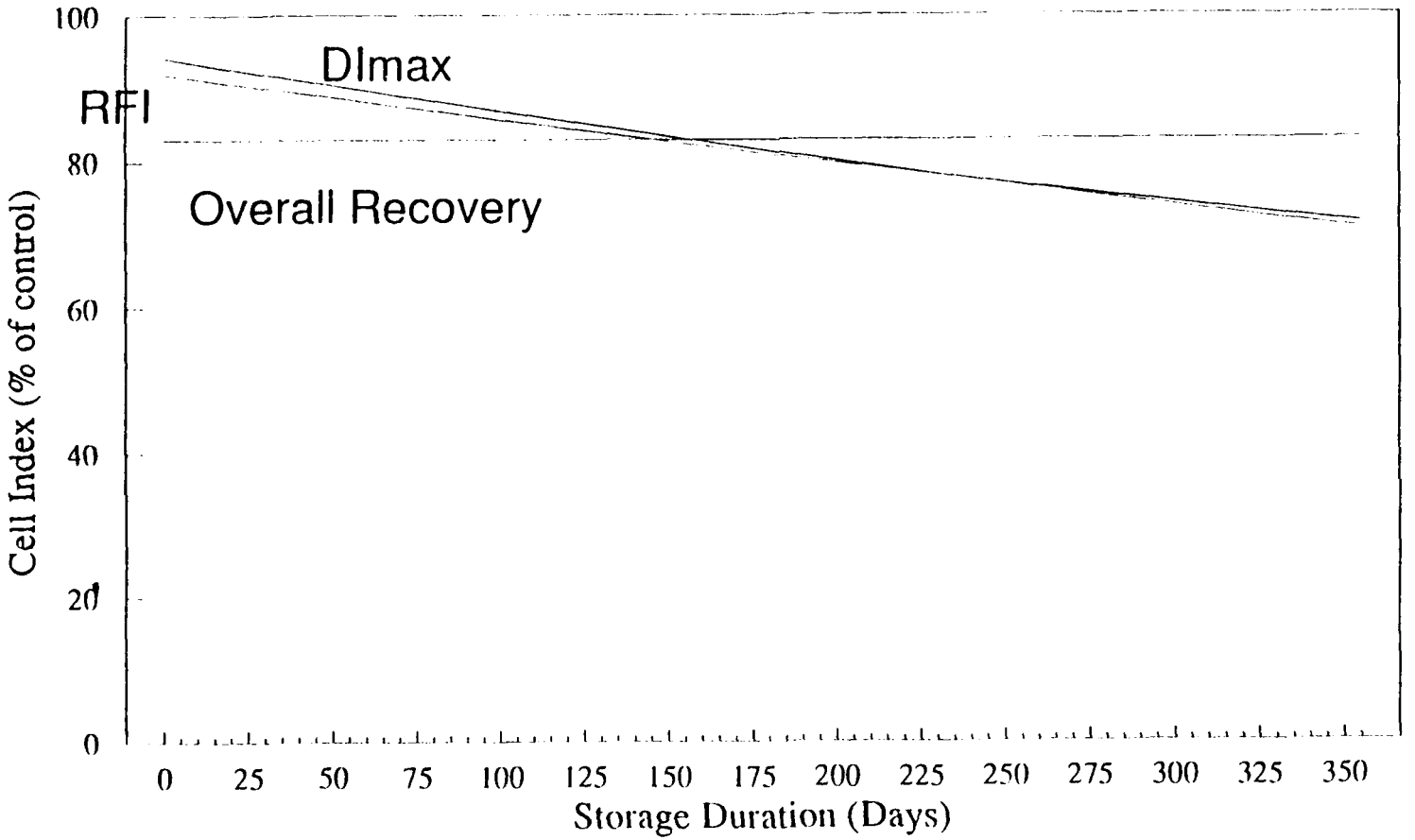

FIGURE 5

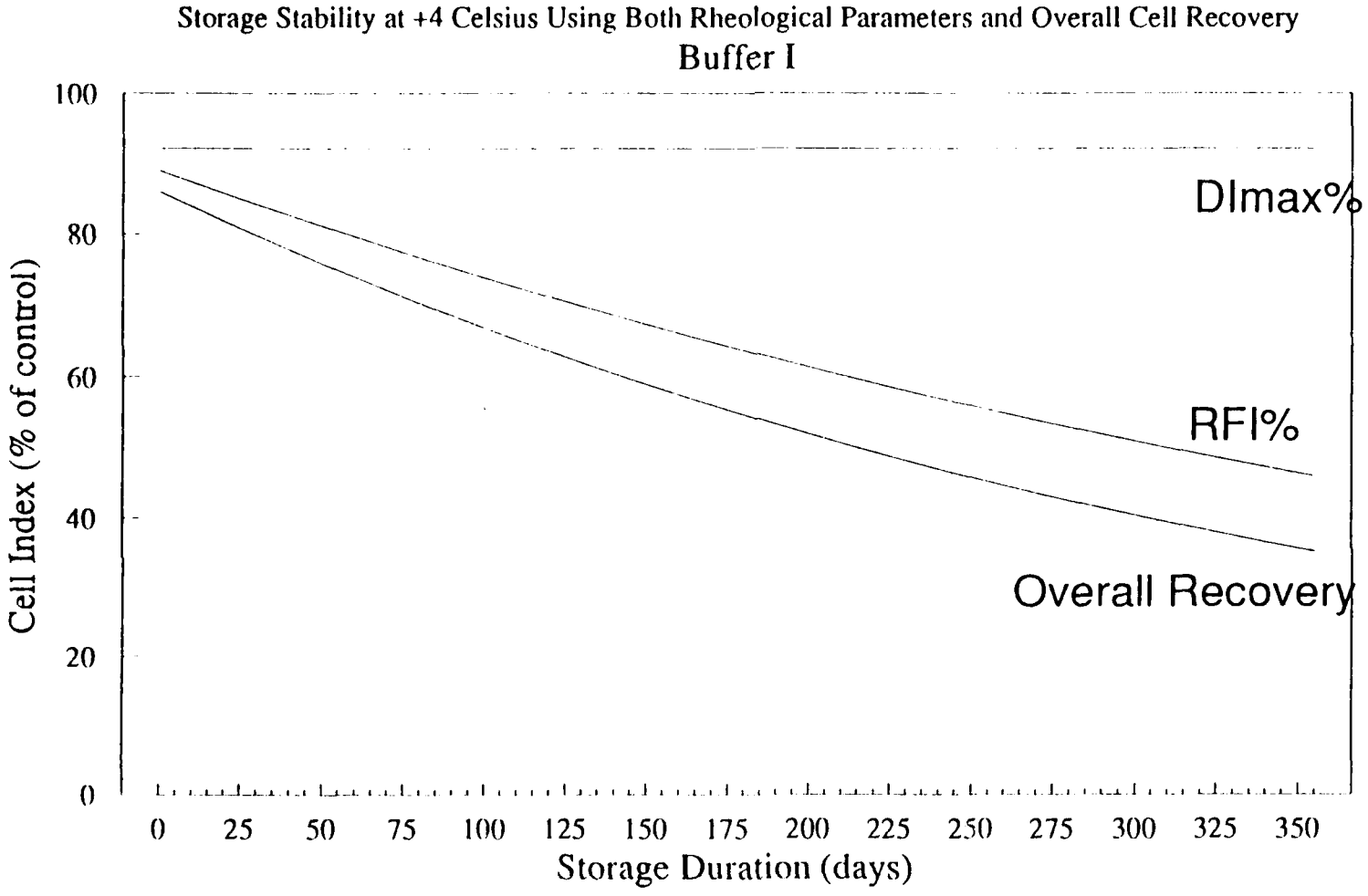
Storage Stability at +4 Celsius Using Both Rheological Parameters and Overall Cell Recovery
Buffer I
Cell Index (% of control)
100
80
60
40
20
0
DImax%
RFI%
Overall Recovery
0
25
50
75
100
125
150
175
200
225
250
275
300
325
350
Storage Duration (days)

Table 1: Effect of storage duration on surface antigens of lyophilized human red blood cells.

A

	Base line Values From Fresh Non-Lyophilized Red Blood Cells			
SAMPLE	ANTI P_1	ANTI N	ANTI Fy^a	ANTI Le^a
JP	3+	0	0	3+
IW	4+	3+	0	0
JE	4+	2+	0	4+

B

	Lyophilized RBC (Day 1)			
SAMPLE	ANTI P_1	ANTI N	ANTI Fy^a	ANTI Le^a
JP	4+	0	0	4+
IW	4+	3+	0	0
JE	4+	3+	0	3+

C

	Lyophilized RBC stored at +4Celsius for 7 days (Week 1)			
SAMPLE	ANTI P_1	ANTI N	ANTI Fy^a	ANTI Le^a
JP	4+	0	0	3+
IW	4+	2+	0	0
JE	4+	1+	0	4+

D

	Lyophilized RBC stored at + 4Celsius for 21 days (Week 3)			
SAMPLE	ANTI P_1	ANTI N	ANTI Fy^a	ANTI Le^a
JP	4+	0	0	3+
IW	4+	3+	0	0

E

	Lyophilized RBC stored at +4 Celsius 28 days (Week 4)			
SAMPLE	ANTI P_1	ANTI N	ANTI Fy^a	ANTI Le^a
IW	4+	3+	0	0

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